DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA.* I. EFFECT OF THE MEDIUM ON FERTILIZING POWER

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Introduction

The investigations in sperm physiology may be roughly divided into two principal aspects. First, there is the problem of the role of the sperm cell in fertilization. Second, there is the problem of the survival of spermatozoa as a fundamental condition for the survival of the species. These two aspects each have their own long lists of investigations.

The study of the sperm cell in fertilization has produced one outstanding theory. This is the Fertilizin Theory of Lillie (1914). The course of investigations, past and recent, shows that this theory, although not completely confirmed and probably in need of modification, has been found useful by many workers in the field. According to the theory, the male germ cell is the carrier of a substance, the "sperm receptor," which is functional in the fertilization process. This substance is thought to combine with "fertilizin," an egg secretion. The complex of sperm-receptor-fertilizin then reacts with an "egg receptor" to form a three-way complex in the egg. The formation of this ternary complex initiates the fertilization reactions of the egg.

The study of sperm senescence, in contrast to the above, has yielded results which are, at best, unsatisfactory. Gray (1928a and b), who investigated the changes in metabolism of sperm under various conditions, reported that sperm in highly concentrated condition have a very low rate of respiration. If diluted, the sperm show a burst of metabolic activity. The greater the dilution, the more intense is this burst of action, although of shorter duration. Gray advanced the hypothesis that a large part of the sperm cell's internal supply of fuel was used up in the first burst of energy, so that the greater its intensity, proportionately shorter became the life of the spermatozoön. The initial burst of activity was in turn determined by the available "free space" in which the sperm cell could move, that is, by the dilution. In the limited space available to each cell in the concentrated suspensions, the sperm cell was only incompletely activated, and, hence, its life was prolonged.

This explanation cannot be applied without certain limitations. If it were, a single spermatozoön placed in an infinitely large volume of diluent would end its metabolism instantly. Further, "mechanical crowding" as an explanation is applicable only to the translatory or vibratory activity of the sperm and not to the respiratory activity. With all the known variables, such as oxygen and carbon dioxide tensions, rigidly controlled, Gray's evidence shows that when "free space" is available the rate of sperm respiration increases. "Mechanical crowding" is thus

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not an explanation for the changes in respiratory rate but a description of the conditions under which the respiratory rate is low. That is, it is logical to state that sperm are quiescent because they are forced to be immobile, but it is not logical to state that sperm respire at a high rate because they are no longer forced to be immobile. Such a statement has implications of teleology. There must exist an unknown factor which, under conditions of dilution, brings about the increased respiration of sperm. Undiluted sperm, therefore, must be a system composed of the cells plus the unknown factor. Dilution of the system, not the dilution of cells alone, brings about the respiratory activity of the spermatozoa.

The foregoing review shows that Lillie's fertilization studies have indicated the existence of a substance that determines the fertilizing power of the sperm cell. The review shows, too, that Gray's work has neglected one variable, the sperm cell medium, or a factor in that medium which affects the duration of metabolic activity of the sperm cell. It is the purpose of this research to present evidence for the existence of a single factor that influences the conservation of fertilizing power by sperm and the respiratory activity of sperm. The work is presented in two sections, the first section dealing with the fertilizing capacity of sperm, and the second with the respiratory activity of sperm.

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Materials and Methods

The materials used in the series of experiments to be described were the germ cells of the Atlantic sea-urchin, *Arbacia punctulata*. The general methods and precautions outlined by Just (1939) were followed carefully. To obtain the germ cells, the urchins were thoroughly washed in running sea water and running tap-water, after which they were dried carefully with clean cheese-cloth. A cut around the oral region disclosed the sex of the animal. If male, the sperm exuding from the genital pores were received in a dry stender dish; if female, the animal was allowed to shed the eggs into a stender dish filled with sea water.

The sperm suspensions for the earlier experiments were made according to the "drop" method of Lillie (1913). For greater precision in later experiments, sperm were "packed" by centrifugation at 3500 r.p.m. for 30 minutes. These packed sperm cells were drawn into a calibrated capillary tube. The tip of the capillary was wiped clean, and the contents were used to make the sperm suspension. The capillary was calibrated by taking up the same volume of re-distilled mercury and weighing the mercury accurately.

As a check on the constancy of this method, sperm counts were made. A unit quantity of packed sperm was suspended in one cc. of sea water, shaken thoroughly, and 0.01 cc. of Bouin's fixative added. This suspension was diluted one hundred times, and the number of sperm present counted in a haemocytometer chamber. The results are given in Table I, and it was found that the greatest deviation from the average was in the order of 6 per cent, a constancy not attainable by the "drop" method.

The seminal fluid used in the experiments was collected simply by drawing off the supernatant fluid from the packed "dry" sperm after centrifugation.

The egg suspensions were made by washing the eggs several times in sea water and allowing them to settle in the dish by force of gravity. Equal samples of the

TABLE I

Sperm count, using 0.00155 cc. packed sperm per cc. of sea water, diluted 100 times

Suspension	Number squares counted	Total counted	Average number per sq.	Conc. of packed sperm per cc.
No. 1	32	386	12.0	3.06×10^{12}
No. 2	32	346	10.8	2.76×10^{12}
No. 3	32	379	11.8	3.02×10^{12}
No. 4	32	360	11.2	2.86×10^{12}
No. 5	32	390	12.1	3.09×10^{12}
No. 6	32	359	11.2	2.86×10^{12}
Average				2.94×10^{12}

settled eggs were diluted in varying amounts of sea water, mixed to give homogeneity, and aliquots were removed with a calibrated pipette. From the number of eggs present per unit length, the total number of eggs could be calculated. One drop of a suspension of suitable egg-concentration was placed in 5 cc. of sea water. Generally, the number of eggs in one insemination test was 750–1000. As Lillie (1915a) had shown, variations of this order in the total number of eggs used in the inseminations do not affect the final results appreciably. Since a fresh egg suspension was used for the insemination tests at any one time, the tests at two different times used different suspensions whose concentrations varied somewhat, so that the results were possibly not comparable. Those tests run at any one time used the same egg suspension, and, therefore, the results were comparable to each other.

For the insemination, a unit quantity of the sperm suspension in a pipette was carefully squeezed out over the eggs, and the whole dish gently and uniformly stirred. For determination of fertilizing power, the percentage of eggs activated was calculated by counting a minimum of 200 eggs.

Widely diverging types of experiments were made in the course of this investigation, each type entailing its own methods and techniques. Because of this, other methods and techniques will be described in connection with particular experiments. Each typical experiment to be presented in the following section was one of a minimum of five experiments all giving similar results.

EXPERIMENTS AND RESULTS

The seminal fluid factor and the survival of sperm

Past researches have shown that sperm in the undiluted condition freshly-exuded from the testes are immobile, and that the sperm manifest intense activity upon dilution with sea water. Subsequently, the fertilizing power of the sperm cells declines sharply and within a relatively short time. Workers in the past had diluted sperm fresh from the testes with sea water. Since the medium seemed to be a variable in this type of dilution, and since sperm cells in the testes were suspended in a liquid medium, a factor influencing the fertilizing capacity of sperm cells was sought in the seminal fluid.

To examine the effect of the seminal fluid on the fertilizing power of sperm, a series of experiments was done using sperm suspensions of the same concentration in seminal fluid and in sea water. These suspensions were then tested at different time intervals for their fertilizing power. In the experiment shown in Table II, a 0.4 per cent sperm suspension (according to the terminology of Lillie) was used. One drop of the suspension was used to inseminate 750–1000 eggs. The formation of the fertilization membrane was used as the index of activation of the egg.

The effect of the seminal fluid in promoting the survival of the sperm was apparent even after five hours, and after 12 hours, when the sperm in sea water were completely non-functional, a large number of those in the seminal fluid were still capable of bringing about activation. At each test, microscopic observation revealed that the per cent activation of eggs was approximately directly proportional to the number of motile sperm.

TABLE II

Activation of eggs by sperm suspensions of 0.4 per cent concentration in seminal fluid and sea water

Medium	Per cent activation					
	10 a.m.	11 a.m.	3 p.m.	5 p.m.	8 p.m.	10 p.m.
Sea water	100	100	46	22	0–2	0
Sem. fluid	100	100	99	100	99	95

The maintenance of fertilizing power of the sperm cells was a function specific for the seminal fluid. Experiments were made using the perivisceral fluid as the suspension medium. The perivisceral fluid was found to have a toxic effect on the retention of fertilizing power by sperm.

It seemed clear that in the seminal fluid an unknown factor was enabling the sperm to retain their fertilizing power for a long period of time. In view of the work of Cohn (1918), a check of the effect of pH became necessary. The pH of the seminal fluid was measured electrometrically with McGinnis' electrode. A number of such measurements showed the pH of seminal fluid to vary between 7.6 and 7.9.[1] Experiments were done comparing the survival of sperm in seminal fluid and sea water acidified to the same pH as the seminal fluid sample.

In the same experiments, another chemical property of the seminal fluid was investigated, namely, the heat-sensitivity. A sample of the seminal fluid in a test tube was heated at 100° C. for ten minutes, the seminal fluid allowed to cool to room temperature, and this heated seminal fluid was tested for its effect on the survival of sperm.

The results of experiments are summarized in Table III. The dilution used was one drop of centrifuged sperm to 5 cc. of medium. The pH of this seminal fluid sample was 7.72; the sea water (pH 8.0) was acidified to 7.7 by the addition of 11 drops of 0.1 N HCl to 100 cc. of sea water. All the suspensions were made at 5 p.m.

The results showed that acid sea water maintained the fertilizing power of the sperm only slightly longer than normal sea water and not nearly so long as the

[1] Done by Mr. M. E. Smith, of the MBL staff.

TABLE III

The effects of pH, heated seminal fluid on the survival of sperm, as shown by time measurements of the fertilizing power

Medium	Per cent activation				
	5 p.m.	9 p.m.	10:30 p.m.	4 p.m.	10 p.m.
Sea water	100	100	14	0	0
Sem. fluid	100	100	100	97	73
Heated fluid	100	0	0	0	0
Acid s.w.	100	100	40	2	0

seminal fluid. The heated seminal fluid, on the other hand, had clearly lost the function of promoting the survival of the sperm cells. It was evident that pH was not the effective factor in the seminal fluid and that the effective factor was heat-sensitive.

This heat sensitivity led to the suspicion that the unknown factor was protein. To test this hypothesis, the seminal fluid was saturated with ammonium sulfate. A faintly rose-colored precipitate resulted from this treatment. This precipitate was filtered off, and the residue on the filter paper dissolved in a volume of sea water equal to the original volume of seminal fluid. The sea water containing the residue was then dialyzed against fresh changes of sea water in the refrigerator for 30 hours. The dialyzing membrane was commercial sausage skin (Cenco). This treatment removed the ammonium sulfate. The liquid inside the dialysis bag, essentially an artificial seminal fluid, was then used as the suspending medium for the sperm.

As controls for this experiment, various other media were used to suspend equal concentrations of the same sperm sample. For the first of these, the filtrate of the seminal fluid (seminal fluid minus the precipitated material) was also dialyzed against sea water for the same length of time as the residue solution, and this "dialyzed filtrate" was used as a suspending medium for the sperm. Normal sea water, acid sea water, and natural seminal fluid were also run as controls. The dilution used was one drop of centrifuged sperm to 10 cc. of medium, and the pH was carefully checked in each case.

The results (Table IV) showed that spermatozoa in the "artificial seminal fluid" retained their fertilizing power nine hours longer than did the sperm in sea water. From the data, it was concluded that the seminal fluid factor was precipitable with ammonium sulfate and non-dialyzable. The earlier conclusion as to the negligible effect of pH was confirmed in this experiment.

The idea of the seminal fluid factor's being protein seemed to be borne out and warranted an analysis of the seminal fluid for its protein content, along with determinations of other physical and chemical properties. For determination of protein, Folin's micro-Kjeldahl with direct Nesslerization was used, the solutions being compared in a photoelectric colorimeter. The results showed 2.5 mg. protein per cc. of 100 per cent seminal fluid. The pH of the seminal fluid was found to vary between 7.6 and 7.9 as compared to the pH of sea water, which varied from 7.9 to 8.1.[2] The freezing point of seminal fluid was $-1.715°$ C. as compared to that of

[2] Done by Mr. M. E. Smith, of the MBL staff.

TABLE IV

The effects of various media on the survival of sperm, as shown by insemination tests

Medium	pH	Per cent activation					
		0.5 hrs.	5.5 hrs.	10.0 hrs.	15.5 hrs.	24.0 hrs.	26.0 hrs.
Sea water	8.0	98	92	49	13	0	0
Acid sea water	7.7	98	87	72	68	0	0
Sem. fluid	7.6	100	97	100	100	98	35
Dial. residue	7.8	99	96	98	85	19	4
Dial. filtrate	7.8	93	80	20	5	0	0
Eggs tested		98	98	100	100	98	100

sea water, which was $-1.892°$ C.[3] Chloride analysis showed the sea water to contain 0.508 moles per liter, while the seminal fluid contained 0.590 moles per liter.[4] Analysis for glucose (reducing sugar) showed the seminal fluid to contain less than 10 gamma in 5 cc.

These results suggested as one possibility that the action of seminal fluid on the sperm could be attributed to the osmotic pressure difference between the seminal fluid and the sea water. The demonstrated heat-sensitivity of the seminal fluid factor, however, ruled this possibility as unlikely, as did the prolonged dialysis of the last experiment given, for such treatment would equalize the osmotic pressure of the seminal fluid with that of the sea water.

The difference in chloride content between the seminal fluid and sea water was not considered as a factor in prolonging the fertilizing power of the sperm cells. The prolonged dialysis described earlier would have equalized the chloride concentration of the sea water and the "artificial seminal fluid" of Table IV, yet these two media had markedly different effects upon the sperm cells. Also, the demonstrated heat sensitivity of the seminal fluid factor indicated that it was not chloride.

The effective seminal fluid factor therefore seemed to be protein, but protein, by its presence, would establish a colloidal osmotic pressure which might be the agency acting on the sperm.

In Table IV, it may be noted that the "dialyzed residue" was not as effective as the natural seminal fluid. There are several possible explanations. First, during the prolonged dialysis, some of the protein may have been denatured, a point to be checked in future investigations. Second, the concentration of the factor in the "artificial seminal fluid" was probably not equal to that in the natural medium, due to some loss of protein in handling, and difficulties in volume control in dialysis.

At this point, attention should be called to the fact that still another possibility existed as to the manner in which the seminal fluid functions. This was the question of nutrition of the sperm by the seminal fluid. This question will, however, be taken up in the discussion.

There remained one mode of action of the seminal fluid factor hitherto uninvestigated. The results of the experiments already described validated the as-

[3] Done by Dr. Jay A. Smith, of the MBL staff.
[4] Done by Mr. J. Weissiger, of the MBL staff.

sumption that the seminal fluid factor acted in some manner upon the surface of the sperm cells.

Observations made during attempts to measure sperm activity in a capillary tube showed spermatozoa to be positively thigmotropic to glass surfaces. At the instant of contact, the spermatozoön lost a large part of its activity and rotated slowly about its point of contact. The observation seemed to show the presence of a surface active substance on the head of the spermatozoön. This fact, previously observed by Buller (1902), led to the following experiment.

Three suspensions of sperm of equal concentration were made in sea water. Suspension No. 1 was left untreated. Glass powder was added to suspensions No. 2 and No. 3. All three suspensions were shaken simultaneously and placed in the refrigerator, where the powdered glass was allowed to settle for three hours. Insemination tests were run to determine the relative sperm populations in these three suspensions. Qualitative microscopic observations on sperm population were also made at each dilution of the original suspensions as a check.

As shown in Table V, the results indicated that the sperm population in the second and third suspensions was greatly reduced, a result confirmed by microscopic observation. It was possible that the glass powder injured a large part of the total sperm population, but the absence of significant numbers of injured sperm seemed to indicate that the glass powder removed the missing sperm by adhesion.

TABLE V

Activation of eggs by progressive dilutions of sperm suspensions treated with glass powder as compared to untreated sperm suspension

Suspension	Undiluted	1:1 Dilution	3:1 Dilution
No. 1	100	100	100
No. 2	96	61	27
No. 3	100	75	35

A similar experiment was made to test the surface activity of seminal fluid protein, since the proposed surface-action implied the identity of sperm-surface-substance and seminal fluid protein. A sample of seminal fluid was divided into three portions. Portion No. 1 was left as the untreated control. Glass powder was added to portion No. 2, the portion shaken thoroughly, and the glass powder filtered off with Whatman No. 5 filter paper. Portion No. 3 was shaken three times, each time with fresh glass powder and filtered free of glass each time. These seminal fluid portions were then used to make sperm suspensions of equal concentration and tested for the maintenance of the fertilizing power. The results are given in Table VI.

Clearly, the glass powder removed the sperm-longevity factor from the seminal fluid, so that seminal fluid protein, too, seemed to be surface-active on glass. Although the experiments of Tables V and VI did not completely establish the identity of the seminal fluid factor and the substance on the surface of the sperm, they did show that both substances were apparently surface-active.

In furtherance of this line of thought, experiments were made to learn whether sperm in sea water gave off their surface substance into the surrounding medium.

TABLE VI

Removal of the factor from seminal fluid with glass powder

Medium	Per.cent activation						
	0.0 hrs.	4.0 hrs.	9.5 hrs.	12.0 hrs.	14.5 hrs.	24.0 hrs.	28.0 hrs.
Sea water	100	100	78	65	40	0	0
Portion No. 1	100	100	100	100	100	100	95
Portion No. 2	100	100	100	80	54	5	0
Portion No. 3	100	99	1	0	0	0	0
Eggs tested	100	100	100	100	100	100	100

In one type of experiment, a heavy suspension of sperm in sea water was allowed to stand for several hours. The sperm were then removed by centrifugation and the supernatant fluid tested as a sperm medium. In another type of experiment, the above procedure was repeated several times, the supernatant fluid used to support a fresh sample of sperm after each centrifugation. After the final centrifugation, the supernatant fluid was tested for its effect on fresh sperm. In all cases, the results were negative. Such "sperm washings" had neither a detrimental nor favorable effect on the maintenance of the fertilizing power of the sperm.

There remained one other point of investigation in the survival time of spermatozoa. Observations had shown that seminal fluid protein, even in low concentration, was effective in maintaining spermatozoa. Gray (1928a) had postulated a "mechanical crowding" effect as the primary factor in the survival of sperm. Since he used "dry" sperm, which was composed of about 60 per cent seminal fluid, there arose the possibility that the longer survival of the more concentrated sperm had as its cause, not "mechanical crowding," but the larger amounts of seminal fluid protein carried over in the "dry" sperm. A test of this possibility followed.

A sperm suspension in seminal fluid was made by suspending 0.025 cc. of packed sperm in one cc. of seminal fluid. A second suspension was made by taking 0.2 cc. of the first suspension and adding it to another one cc. sample of seminal fluid. This serial dilution was repeated twice more, to make four sperm suspensions, all in seminal fluid. The operation was carried out quickly, the last suspension made within a minute of the first. The final concentrations of the four suspensions were, in Lillie's terminology, approximately 5 per cent, 1 per cent, 0.2 per cent, and 0.04 per cent, since packed sperm contained approximately twice the amount of sperm per unit volume as did the "dry" sperm used by Lillie. The insemination tests were made at the same dilution, each of the more concentrated suspensions being diluted to the lowest concentration of 0.04 per cent. One drop of this final suspension was used to inseminate the eggs. The results are given in Table VII.

A study of these results as compared to those of Gray showed that, even though Gray's results might be partly explained as the action of seminal fluid protein, "mechanical crowding" did seem to play a part in determining the life-span of the spermatozoa. However, it may be pointed out that this "crowding effect" seems to be non-linear in relation to the concentration, and is most apparent at extreme dilutions.

TABLE VII

The effect of concentration on the survival of sperm in seminal fluid

Suspension concentration	Per cent activation					
	0.5 hrs.	4.0 hrs.	7.5 hrs.	18.0 hrs.	23.0 hrs.	30.0 hrs.
5 per cent	88	90	78	77	85	91
1 per cent	81	85	83	79	83	72
0.2 per cent	90	92	84	80	49	13
0.04 per cent	92	95	86	45	10	0

The seminal fluid factor and its role in fertilization

In the course of the preceding experiments, sea water suspensions of sperm used to test the eggs showed a contrasting behavior as to fertilizing power. The individual spermatozoön in seminal fluid appeared to have a greater fertilizing power than the spermatozoön in sea water. An experiment was devised to investigate this more closely.

A volume of 0.025 cc. of packed sperm was suspended in one cc. of seminal fluid. Immediately after the suspension was made, one drop of the suspension was used to inseminate approximately 1000 eggs. Serial dilutions were made as for the previous experiment, but as each new suspension was made, one drop was used to inseminate approximately 1000 eggs. A sea water control was run, dilution and inseminations being made in the same way (Table VIII).

TABLE VIII

A comparison of the fertilizing power of sperm in seminal fluid and sperm in sea water

Medium	Per cent activation			
	1st dilution	2nd dilution	3rd dilution	4th dilution
Sea water	100	97	37	12
Sem. fluid	100	99	100	81

The results proved that there was a strong difference in the fertilizing power of the sperm cells in seminal fluid as compared to those cells in sea water. This difference became even more pronounced when the original concentrated suspensions were allowed to stand for ten hours, as shown in Table IX. Only the most concentrated suspensions in the seminal fluid and the sea water were kept. The dilutions were made anew.

The interpretations of these results were rather complex and will be discussed in a later section.[5]

The apparent increased fertilizing power of the sperm in the seminal fluid indicated that seminal fluid factor might be directly concerned with the fertilization process. It was recalled that Lillie (1915) had given as one of the criteria for the "sperm receptor" the power to "bind" agglutinin from the egg. Lillie meant by this

[5] See page 175.

TABLE IX

A comparison of the fertilizing power of sperm in seminal fluid and sea water after 10 hours

Medium	Per cent activation			
	1st dilution	2nd dilution	3rd dilution	4th dilution
Sea water	100	86	7	0
Sem. fluid	100	100	98	79

that if the agglutinin were treated with the "sperm receptor" solution (here the seminal fluid, presumably), the action of the agglutinin on the sperm would be greatly reduced. This experiment was done, with the expectation that, if the seminal fluid factor and the "sperm receptor" were one and the same, the agglutinating action of the egg secretion would be reduced.

A series of dry watch glasses was arranged. In the first, two drops of seminal fluid and two drops of egg-water were thoroughly mixed. Two drops of this mixture were then removed to the next watch glass and diluted with two drops of sea water. This treatment was repeated down the series. For the control, sea water was used instead of seminal fluid. For the test, a drop of a standard sperm suspension (0.00155 cc. packed sperm per cc. of sea water) was placed in the watch glass, out of contact with the mixture. The watch glass was then placed under the objective of the microscope, the two liquids (sperm suspension and sea water-seminal fluid mixture) shaken together, and the reaction of the sperm noted. In the following table, + indicates a positive agglutination, − a negative agglutination, and ± uncertain. The number of + symbols indicates the intensity of the reaction.

TABLE X

The agglutination reaction induced by dilutions of egg-water-seminal fluid mixtures, as compared to those induced by egg-water-sea water mixture of the same dilutions

Dilution	Sea water egg-water	Sem. fl. egg-water
1	+++	++++
1/2	+++	++++
1/4	+++	+++
1/8	++	+++
1/16	+	++
1/32	±	+
1/64	−	+
1/128	−	±

Instead of having its action on the sperm reduced, the results revealed that egg-water treated with seminal fluid had, if anything, a more powerful agglutinating power than the sea water-treated egg-water. In any event, the agglutinating power was not reduced. The only conclusion possible from these results seemed to be that the seminal fluid factor was not the "sperm receptor" of Lillie.

However, the data given indicated that the sperm reaction in the seminal fluid-egg-water mixture was more intense than the corresponding reaction of sperm in the egg-water sea water mixture. This phenomenon was put to a quantitative test.

Standard suspensions of sperm were made in seminal fluid and in sea water. The concentration was 0.00155 cc. packed sperm per cc. of medium. These suspensions were allowed to stand at room temperature (25° C.). At intervals, a drop from either one or the other of the suspensions was placed on a watch glass, out of contact of a mixture of one drop of egg-water and two drops of sea water. The liquids were shaken together under the microscope, and the time of agglutination (from onset to reversal) was taken with a stop-watch. The results are summarized in Table XI.

Table XI

The agglutination time of sperm suspended in seminal fluid as compared to that of sperm suspended in sea water

Time Tested	Agglutination time in seconds	
	Sperm in sea water	Sperm in seminal fluid
p.m.		
3:00	90	120
3:06	63	69
3:10	61	115
3:18	82	98
3:40	49	111
3:44	53	95
3:50	47	91
3:57	63	90
4:05	61	97
4:40	91	86
4:50	86	75
5:15	34	120
5:30	71	115
7:30	75	76

The data show that, on the average, the sperm in the seminal fluid remained agglutinated for a longer time than the sperm in sea water. Although the results showed wide variation, the contrast between the two sperm suspensions was quite striking. From the results, it seemed reasonable to conclude that seminal fluid had changed the sperm surface in such a way as to bring about a stronger reaction with agglutinin.

Discussion

The seminal fluid factor and sperm motility

Gray (1928a) observed that the motility of the sperm of *Echinus miliaris* was in no way impaired when suspended in seminal fluid, and he stated conclusively that the seminal fluid possessed no chemical or physical properties inhibiting sperm motility. He prepared the seminal fluid, which he called "testicular plasma," by strong centrifugation of the "dry sperm," the same method employed in this investigation.

The experiments and observations of the present study confirm Gray. The earlier results of the work, given in a preliminary note (Hayashi, 1940), showed that the sperm of *Arbacia punctulata* were motile in seminal fluid, with an intensity of movement at least equal to that exhibited by sperm in sea water. Moreover, this motility persisted for a longer time in the former medium. That sperm are active in seminal fluid was confirmed by respiration studies (results to be given in a subsequent report), for it was found that the respiratory activity of sperm was maintained

at a higher level for a longer time in seminal fluid than in sea water. Therefore, it may be stated conclusively that sperm cells of *A. punctulata* and *E. miliaris* are fully active in seminal fluid.

The observations and conclusions of Southwick (1939a) were found to be in conflict with these results. This worker found that sperm of *Echinometra subangularis* were immobile when suspended in the seminal fluid of the same species. He concluded that there was present in the seminal fluid a substance which inhibited the activity of the sperm.

Hartman (1940) and Hartmann, Schartau, and Wallenfels (1940) confirmed Southwick on the presence of the inhibiting factor not only in the seminal fluid, but also in the sea water that had contained large numbers of spermatozoa. Their work, however, was done with the sperm of *Arbacia pustulosa*. In addition to confirming Southwick, Hartmann et al. stated that the function of the inhibiting factor was to neutralize echinochrome A, a sperm-activating substance from the egg.

For several reasons, the conclusions of these workers do not seem to be justified. In the first instance, Southwick's own observations reveal that freshly-exuded "dry sperm" possess an intense vibratory activity, an apparent contradiction to his own conclusion. This activity is lost after a few minutes. A number of investigators have published observations pertinent to these phenomena. Thus, Harvey (1930) showed that sperm of *Arbacia punctulata* in oxygen-free sea water were immobile; when oxygen was introduced the sperm regained their motility. Lillie (1913) demonstrated that sperm of Nereis and Arbacia lost their motility in the presence of carbon dioxide. Dungay (1913), using Nereis and Arbacia, Fuchs (1914) with *Ciona intestinalis,* and Cohn (1918) with *A. punctulata* proved that acid media had a deleterious effect on sperm. Finally, Carter (1931) working with *Echinus esculentus* and *Echinus miliaris,* and Taylor and O'Melveny (1941) with *Strongylocentrotus purpuratus* and *Lytechinus anamesus* obtained experimental proof that acid conditions lowered the respiratory activity of sperm.

In view of the results of these investigators, the brief activity of the sperm noted by Southwick seems to be attributable to the newly-made contact of the sperm with oxygen upon shedding. The subsequent inactivation of the sperm has its probable explanation in the acid conditions induced by the carbon dioxide production of the sperm.

Furthermore, the papers of Southwick and the Hartmann school yield no figures on the pH of the media used by these workers, nor do their texts give any evidence that this factor had been controlled. In addition, the conclusions of Hartmann et al. concerning the effect of echinochrome have not been confirmed by the experiments of Tyler (1939b) and Cornman (1940, 1941). The former worker found that neither echinochrome nor spinochrome would stimulate the respiration of sperm of *S. purpuratus.* The latter showed that crystalline echinochrome did not increase the motility of the sperm of *A. punctulata.* The paradoxical results of Tyler and Cornman as opposed to Hartmann et al. may be attributed to species difference. However, it is clearly possible that echinochrome does not activate sperm. The non-existence of a sperm-activating function by echinochrome seems to weaken the argument for the existence of a substance neutralizing that activating factor.

Because of these considerations, the concept of a sperm-inhibitor in the seminal fluid seems to be questionable. In the light of parallel experimental results as re-

gards sperm motility and respiratory activity (Hayashi, unpublished), it is concluded that there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*. This conclusion does not deny the inhibiting effects of hydrogen ions, the influence of which on the increase of the life-span of the sperm has been shown to be insignificant. To restate the conclusion: excluding the hydrogen ion factor, there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*.

The seminal fluid factor in its relation to the activating capacity of the sperm

Various experiments have proved that spermatozoa suspended in seminal fluid retain their capacity to activate eggs longer than sperm cells suspended in sea water (Tables II, III, IV). The factor in the seminal fluid responsible for the effect is not found in the perivisceral fluid, the factor is not the pH of the medium, and the factor is heat-sensitive (Table III). The seminal fluid factor is also non-dialyzable and precipitable with ammonium sulfate (Table IV). On the basis of these results, it may be tentatively stated that the seminal fluid factor is protein. However, the usual protein tests have not been made, so that this conclusion cannot be drawn with any finality, even though the conclusion is strongly supported by positive micro-Kjeldahl analyses indicating protein content in the order of 2.5 mg. protein per cc. of seminal fluid.

The seminal fluid factor, if protein, may act on the sperm cells in several ways. The factor may serve as a source of nutrient for the sperm, it may act on the sperm through the agency of the colloidal osmotic pressure which its presence establishes in the seminal fluid, or it may act through adsorption on the sperm surface. It is necessary to consider these possibilities carefully, if the mechanism of the action of the seminal fluid factor is to be clarified.

The possibility of the seminal fluid factor's acting as a nutrient will be taken up more fully in a later publication on the effect of the seminal fluid on the respiration of sperm. The statement can be made here that these studies indicate that the factor does not act as a nutrient for the sperm. Likewise, the probable protein nature of the factor argues against the idea of nutrition, for the large size of the molecule would prevent its absorption by the sperm. The fact that seminal fluid contains no reducing sugar is further support for the belief that the seminal fluid affords no nutritive elements for the sperm cells.

The question of the effect of colloidal osmotic pressure in prolonging the functional life of the sperm cell is unsettled. Although the further experimental results on the surface activity of seminal fluid substance validate the conclusions drawn, it is admitted that the effects of colloidal osmotic pressure on sperm longevity is still an open question.

The experimental results given in Tables V and VI constitute support for the idea of surface-action of the seminal fluid factor. The data show that both the sperm surface and the seminal fluid factor are surface-active on glass, and they indicate the possible identity of the sperm-surface-substance and the seminal fluid factor.

The foregoing considerations point strongly to the conclusions that the seminal fluid factor is protein and that it is present both in the seminal fluid and on the sperm surface. Since the seminal fluid factor enables the sperm to retain their fertilizing function, it seems logical to infer that the seminal fluid protein plays a part directly in the fertilization process. The data of Tables VIII and IX give support

to this idea. The experiment of Table VIII reveals the fact that, with the same amounts of sperm, a higher percentage of activation of eggs is achieved by sperm in seminal fluid than by sperm in sea water. Since the experiment was so arranged that the insemination tests were made immediately after the mixing of each solution, the possibility that a large number of sperm in the sea water were immobilized seems unlikely. The conclusion most compatible with the results is that the individual spermatozoön in seminal fluid possesses a greater fertilizing capacity than his fellow in sea water. The mere act of dilution in sea water, therefore, seems to have removed a large part of the activating substance from the surfaces of the sperm cells, reducing their individual activating power.

The idea of variation of the activating power of the individual sperm cell was first expressed by Glaser (1915). He diluted sperm serially in sea water, and found that several sperm cells were required to activate one egg cell, even though only one spermatozoön was required to bring about the biparental effect. Lillie (1915) found that when he used the same method of dilution as did Glaser, the fertilizing power of the suspension was far less than an equal concentration of sperm in a suspension diluted in one step. Although these two workers disagreed in their conclusions, their results point to the validity of Glaser's interpretation, which is confirmed in the present study.

Table IX shows the relative fertilizing powers of suspensions in sea water and seminal fluid after they had been aged for 10 hours. If the results are compared to those of Table VIII, it will be seen that the fertilizing capacity of the seminal fluid sperm suspension was not affected by the aging period but that the fertilizing capacity of the sea water suspension was markedly reduced. There are two possible explanations for the enhanced difference in the activating power of the two suspensions, both of which probably contribute to the effect. It is possible that in the 10-hour aging period, large numbers of the sperm cells in the sea water suspension were immobilized, so that they could not penetrate the jelly envelope surrounding each egg. Thus, the number of sperm cells making actual contact with the egg surface was reduced. The final result would be a decreased percentage of activation. The second possibility is the conclusion derived from the analysis of Table VIII, namely, that a substance functional in activation was removed from the sperm surface. During the 10-hour period, this removal presumably continued, so that the activating power of the individual sperm cell was further reduced. Therefore, even if all the spermatozoa remained motile and capable of making contact with the egg, more sperm cells per egg would be required for activation, and the end results would be a decreased percentage of activation. The experiment, therefore, tends to support the idea of an egg-activating substance on the sperm surface, and, also, shows the close relationship between the motile activity and the activating power of the sperm cell.

Many investigators have postulated the existence of a substance on the surface of the sperm and considered that it was protein in nature. Buller (1902), from observations of the sperm of various echinids, reported that the sperm surface was surface-active, not only on glass, but also on air bubbles. Lillie (1913) discovered that in the presence of egg secretions, the male germ cells of Arbacia and Nereis became agglutinated. He concluded that the surface of the sperm cell was "sticky." The marked similarity of the agglutination phenomenon to an immunological reac-

tion may be taken to be a strong indication for the protein nature of the responsible agent on the surface of the sperm cell.

More direct evidence came from the work of Popa (1927). Using histochemical technique, this worker concluded that the surfaces of Nereis and Arbacia sperm were covered with a layer of lipo-protein.

Mudd, Mudd, and Keltch (1929) investigated the surface charge of the sperm cells of various echinids. Using the cataphoresis chamber, they reported that the sperm surface was negatively charged. This negativity they found to be increased after agglutination with egg-water. They concluded that their method made possible the detection of substances on the sperm surface.

Henle (1938) and Henle, Henle, and Chambers (1938) found that heat-sensitive antigens existed on the surface of sperm heads. Their work was done with mammalian sperm. Tyler and O'Melveny (1941) obtained rabbit anti-serum by injection of whole sperm of *S. purpuratus* and *L. anamesus*. The anti-serum was found to agglutinate the sperm of these species. These immunological studies again pointed to the protein nature of the sperm-surface-substance.

The evidence cited is not, perhaps, a complete list. The investigations provide enough experimental data, however, to warrant the tentative conclusion that the sperm-surface-substance is protein in nature.

This sperm-surface-substance and the seminal fluid factor may possibly be identical (Tables V and VI). A strong indication of identity could be established if it were shown that the seminal fluid factor alone can activate eggs. Experiments have been started to investigate this possibility, but as yet no conclusive results have been obtained. Comparable work in this direction has not been done. The effect of protein extracts on egg surfaces was investigated by Favilli (1932) and by Hartmann, Schartau, and Wallenfels (1940), while Sampson (1926) reported the activation of eggs by dialysates and filtrates of sperm suspensions. Since her activating factor was dialyzable, and therefore non-protein, it cannot be compared to the seminal fluid factor. In addition, the remarks of Just (1922, 1928, 1929a and b) criticizing the auto-parthenogenesis of Glaser (1914) and Woodward (1921) are equally applicable to the work of Sampson.

Another possible method of establishing the identity of seminal fluid protein and the sperm-surface-substance is to obtain rabbit anti-serum by the injection of seminal fluid. If the anti-serum thus obtained had the power to agglutinate sperm, the results would constitute substantial evidence for the argument that seminal fluid protein and protein on the sperm surface were the same substance. The experiment, however, was not done.

The identity, therefore, is not established, although there is some evidence in this direction (Tables V and VI). Aside from this point, however, there are experimental results throwing light on the origin of and possible relation between the seminal fluid factor and the sperm surface substance. It would be interesting to know whether these substances are secreted by the sperm cells or not, and whether the sperm-surface substance establishes the seminal fluid substance by passing off into the seminal fluid, or whether the seminal fluid substance establishes the sperm-surface substance by adsorption on the sperm surface.

Numerous investigators have reported that sea-urchin sperm give off a substance into the surrounding sea water (Lillie, 1914 and 1915; Southwick, 1939; Hartmann,

1940; Hartmann, Schartau, and Wallenfels, 1940) and that this substance showed protein characteristics (Frank, 1939; Tyler and O'Melveny, 1941). All investigators agreed that the substance showed the properties of "antifertilizin" or the power to "bind" the agglutinin of egg-water so that the agglutinating effect on sperm was reduced.

The question here posed is: does this substance from the sperm cells have the properties of the seminal fluid substance? The point was tested by an experiment in which a sea water suspension of sperm was allowed to stand for several hours, the sperm cells removed by centrifugation, and a fresh sample of sperm suspended in the medium. The results were negative. This medium was not effective in prolonging the fertilizing capacity of sperm, and therefore did not have the properties of the seminal fluid substance.

There is the converse question: Does the seminal fluid substance have the antifertilizin property of the substance coming off the sperm cell? Again, the results were negative (Table X).

The substance coming off the sperm cell does not have the properties of the seminal fluid substance. The results of these experiments indicate, therefore, that a sperm substance does not establish the seminal fluid substance, so that the seminal fluid factor does not have its origin in the sperm cell. The same negative answer as to the origin of the sperm-surface substance cannot be given.

However, the fact that the substance coming off the sperm surface has different properties from the seminal fluid substance signifies nothing regarding the properties of the sperm substance while on the sperm surface. This substance on the surface of the sperm cell is surface-active on glass, as is the seminal fluid substance (Tables V and VI). The seminal fluid factor also enables the sperm cell to maintain its fertilizing capacity longer (Tables II, III, IV) and seems to enhance the fertilizing power of the individual spermatozoön. In addition, the seminal fluid factor affects the surface of the sperm so that the time of agglutination, or the reaction with agglutinin, is increased (Table XI).

These facts point to a tentative explanation of the relation between the seminal fluid factor and the sperm-surface substance. It is possible that a protein substance, originally present in the seminal fluid, is adsorbed on the surface of the sperm cell, thus influencing the fertilizing power of the sperm cell, as well as rendering the surface of the sperm cell surface-active. By this adsorption also, the sperm surface is rendered capable of greater reactivity with fertilizin. The subsequent loss of this substance from the sperm cell results in the loss of fertilizing power and the presence of antifertilizin in the sperm medium. The antifertilizin would be, according to this scheme, a substance changed in certain properties from the original seminal fluid substance.

Summary

1. A factor is present in the seminal fluid of *Arbacia punctulata* which prolongs the fertilizing capacity of the sperm cells of the same species.

2. The factor, which is not found in the coelomic (perivisceral) fluid, is heat-sensitive, precipitated by saturation with ammonium sulfate, non-dialyzable, and surface-active on glass. Since micro-Kjeldahl analysis of the seminal fluid gives positive results corresponding to 2.5 mg. protein per cc. of seminal fluid, it is tentatively suggested that the factor is protein.

3. Seminal fluid has a pH range of 7.6 to 7.9, its osmotic pressure is approximately 10 per cent lower than sea water, and its content of reducing sugar is negligible.

4. In equivalent concentration and immediately after suspension the fertilizing capacity of the individual spermatozoön is greater in seminal fluid than in sea water.

5. Seminal fluid does not contain antifertilizin since it does not neutralize the agglutinating action of egg-water; indeed, this action is intensified.

6. A tentative mechanism, based on the adsorption of a fertilizing substance and its removal from the surface of the sperm cell, is suggested to explain the experimental results. It is proposed that the seminal fluid factor is this fertilizing substance before adsorption and while on the surface of the sperm; it becomes changed upon removal from the sperm surface.

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